Iranian Journal of Virology 2017;11(4): 25-30
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Original Article

Preparation of a pcDNA3 Plasmid for Hemagglutinin-Neuraminidase Gene of Human Respirovirus 3 (HPIV-3)

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Abstract

Background and Aims: Human respirovirus 3 is the second most common agent for respiratory disease in children under five years old. Near 68% of respiratory tract infections belong to this type. However, there is no established vaccine for this virus. The aim of this study was to synthesize a DNA plasmid for HN gene of human respirovirus 3 (HPIV-3) as a first step for the preparation of a vaccine .

Materials and Methods: The HN gene was synthesized and cloned into pcDNA3 plasmid. The plasmids were transformed into COS-7 cell lines. To evaluate the expressed HN protein, the western blotting assay was applied .

Results: The HN protein 36 KD was detected on nitrocellulose membrane using anti-His-tag antibodies .

Conclusions: We prepared a DNA plasmid containing HN gene of human respirovirus 3 to be used for the future studies towards making an effective vaccine. Among hospitalized Iranian children, one-month to five-year, human respirovirus 3 was most frequently observed in respiratory tract infection .

Keywords: DNA plasmid, Human respirovirus, Human parainfluenza, HN gene, cell culture

Introduction

Paramyxoviridae family are enveloped, single-stranded RNA negative-sense, medium-sized viruses (1). The most important viruses that cause infection in children, such as mumps, measles and parainfluenza viruses, belong to this family (2).

Human parainfluenza virus (HPIV) has four types with a 15 kb genome; these viruses were renamed in August 2016 by the international committee on taxonomy of viruses (ICTV) to human respirovirus 1 and 3 in respirovirus genus and human rubulavirus 2 and 4 in genus of rubulavirus (3). After the human respiratory syncytial virus (HRSV), human respirovirus 3 is the second most common agent for respiratory diseases in children under five years old. Generally, 2–17% of respiratory tract infections in hospitalized patients are due to HPIV infections (4-6). A previous study demonstrated that more than 15% of hospitalized patients in Mazandaran, Iran were infected by respirovirus 3 (7).

Serologic examination indicated that 60% of children under two years and 80% under four years were infected with human respirovirus 3 (4). The mortality rate due to respiratory tract infection in children under five years is high worldwide (8). Among human parainfluenza viruses that cause infection in the world, human respirovirus 3 is the most common one; almost 68% of respiratory tract infections belong to this type (9). Annually, in the USA, 23,000 people are admitted to the hospital because of human respirovirus 3; 7% of all hospital admissions due to viral infection,

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reported as human parainfluenza viruses (6, 10).

The virus encodes six structural proteins and at least six mRNA have been detected (11, 12). Two glycoproteins, hemagglutinin-neuraminidase (HN) and fusion protein (F), start the infection by enabling the attachment and entry of the virus into the host cell. Hemagglutinin-neuraminidase is trimer and comprises of two parts hemagglutinin (HA) and neuraminidase (NA) (13, 14). The HA binds itself to sialic-acid-containing cell-surface receptors, while NA leads to the destruction of the sialic acid section (15). In addition, neutralizing antibodies against this glycoprotein of the virus play a protective role. Despite attempts to produce a suitable vaccine, a licensed vaccine is not yet available for human respirovirus 3. Since this virus is common in the respiratory tract of young children, investigation about this virus is necessary for making an appropriate vaccine. Therefor we examined the correctness of the HN protein produced from the desired expression vector in order to produce a DNA vaccine at a later step.

Methods

Preparation of competent cells and cloning of human respirovirus 3 HN gene in pcDNA3 plasmids. A strain of Escherichia coli (Top10F′) cell, which was engineered to have tetracycline resistance marker in its genome, was used in this study. The cells were cultured in LB liquid medium containing tetracycline and were placed in an incubator at 37°C. The growth of cells was monitored and they were harvested at OD 0.6.

The synthesis of HN gene was carried out according to the complete genome of human respirovirus 3 with GeneID: 911961 to amplify the gene in a cloning vector. The plasmids were transformed to E. coli Top10F' cells using heat shock method and cultured in Luria broth medium (1% tryptone, 0.5% NaCl, and 0.5% yeast extract). The medium was solidified with 2% agar for growth on plates. Ampicillin was supplemented at 100 µg/ml. The increased recombinant vectors were extracted (genet bio kit, South Korea) and verified with 1% Agarose gel electrophoresis.

Hemagglutinin gene was amplified by PCR using a primer pair—forward primer 5'-TATAAAGCTTACCACCATGGCAGATCTCC-3' and reverse primer 5'-TATAGGATCC-TTATCAGTGGTG-3'. Kozak sequence and the sites of restriction enzymes HindIII and BamHI were added to the 5' end of the primers. The termination codon was removed from the gene to promote the link of His-tag from the vector. Then, 50 ng of the extracted vectors was used in a thermocycler profile as the first denaturation at 95°C for 5 min, 94°C for 60 s, 60°C for 60 s, 72°C for 90 s, and final extension at 72°C for 10 min. The PCR products were verified by 1% Agarose gel electrophoresis and empty vectors were used as negative controls.

HN expression vector construction and transfection to COS-7 cell lines. COS-7 cells derived from African green monkey kidney tissue were grown in DMEM containing 10% fetal calf serum (FCS), 100 mg/l of streptomycin, and 60 mg/l of penicillin. In each well, 4x104 COS-7 cells were seeded and transfected when reached 60% confluency.

The PCR products and pcDNA3 plasmids were digested with restriction enzymes and the gene was inserted into pcDNA3. The recombinant pcDNA3-HN plasmids were confirmed by PCR, gel electrophoresis (Biospin Extraction Kit, China), and sequencing, followed by introduction into COS-7 cell lines by poly-ethylenimine (PEI) (ExGen 500 in vivo Transfection Reagen), as an efficient non-viral and non-liposomal gene-delivery reagent. For each transfection sample, 3.3 µl of ExGen 500 were added to a dilution of 1 µg of DNA in 100 µl of 150mM NaCl. After vortex for 10 s, it was incubated at room temperature for 10 min and finally 100 µl of this solution was added to each well. Generally, the volume of the ExGen 500/DNA mixture was diluted to 1/10th of the total volume of DMEM as the medium of cell culture. The cells were placed in an incubator at 37 °C for 48h.

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Transfection was controlled by pEGFP plasmid, which codes for the green fluorescent protein (GFP). PcDNA3 plasmid was purchased from ClonTech, Japan. COS-7 and E.coli Top10F' cells were purchased from Institute Pasteur, Iran.

Detection of HN protein. HN protein production was determined by Coomassie-stained SDS-PAGE (12.5%) and by Western Blot. Proteins were transferred to a nitrocellulose membrane for 1 h, washed with 1X PBS (50mM potassium phosphate, 15mM NaCl, pH 7.2) for 5 min and blocked with 2.5% skim milk for 45 min. The membrane was washed three times by 1X TBS-S buffer (Tris-Buffered Saline solution: 50mM Tris-Cl, 150mM NaCl, pH 7.6), anti-His-tag monoclonal antibody diluted to 0.4 mg/ml in 1X TBS-S buffer, was added and incubated at 37 °C for 1 h, washed with 1X TBS-S buffer four times each for 5 min, added to peroxidase- conjugated anti-His-tag antibody diluted 1:15,000 in 1X TBS-S buffer incubated at 37 °C for 1 h, washed with 1X TBS-S buffer, and finally developed with diaminobenzidine (0.5 mg/L) and 1.5% peroxide hydrogen.

Results

The gene was synthesized, analyzed in the gene runner software version 3.05, and subcloned in pcDNA3 plasmid. Insertion of the synthetic gene was verified by gel electrophoresis (Figure 1).

Designed primers were annealed to Kozak and restriction enzyme site sequences. The results of gel electrophoresis, followed by analysis

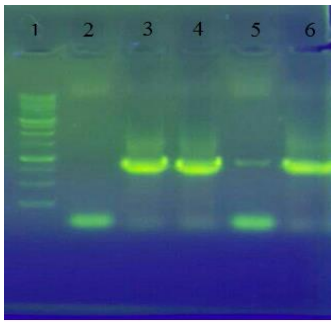


Fig. 1. Detection of HN gene in pcDNA3-HN plasmid by PCR. PCR with primers that annealed to Kozak and restriction enzyme sites sequences amplified a fragment of 992 bp. The length of HN gene was 981 bp (992 in addition to Kozak and restriction enzyme site sequences). Finally, 1kb molecular weight (lane 1), empty plasmid as the negative control (lane 2), and positive samples by 992bp molecular weight are observed in lanes 3–4 and 6.

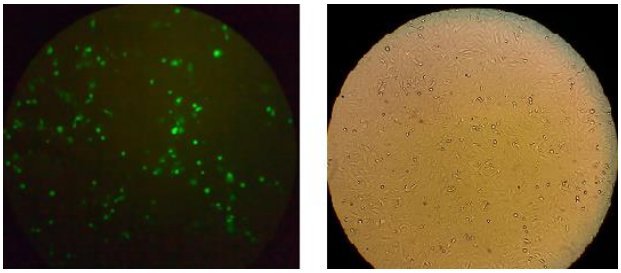


Fig. 2. Confirmation of the expression system in COS-7 cell lines: 1 µg of the pEGFP vectors (left) and empty pcDNA3 plasmids (right) were applied to transfer to COS-7 cell lines. The fluorescent light radiation was due to the presence of pEGFP vector as a positive control for transformation.

(ChromasPro software version 2.1.6) of the achieved sequencing, demonstrate that the constructs were successfully inserted in the expression vectors (Figure 1).

HN gene expression in COS-7 cell lines. After 48 h amplification of recombinant pcDNA3 plasmids in COS-7 cell lines, total lysate content of the cells was subjected to SDS-PAGE. Transfection of the recombinant plasmid was controlled by expression of pEGFP vector (Figure 2). The produced HN proteins were detected by Western Blot assay that used monoclonal antibodies against the His-tag. Based on the gene runner analysis, weight of the protein derived from the synthetic HN gene was 36 KD which was confirmed by electrophoresis (Figure 3).

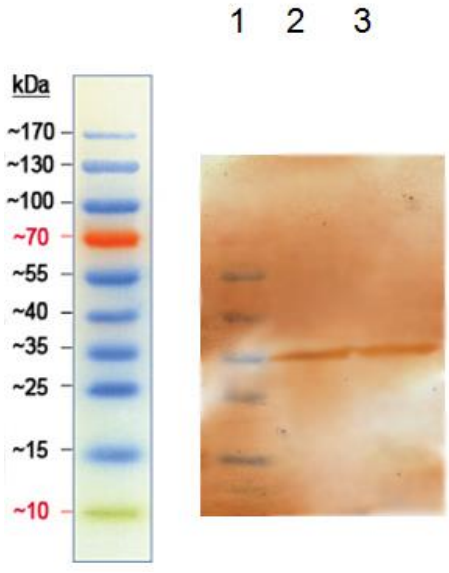


Fig. 3. Western blot for the detection of the HN protein production. The weight of the protein is almost 36 kDa. Lines 2 and 3 confirmed expression of HN by anti-His antibody.

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Discussion

At present, two groups of live-attenuated vaccines are produced against HPIV-3. One group is the chimeric recombinant bovine PIV-3 (BPIV-3) vaccine containing F and HN glycoproteins of BPIV-3 on its surface (16); only 25% of F and HN glycoproteins of BPIV-3 are antigenically related to the same proteins in human respirovirus 3 and could not elicit robust immunity of cells in the human body (17, 18). The other group of vaccines is the wild-type human respirovirus 3 extracted from African green monkey cells at low temperature.

The vaccine, provide a protective response in seronegative infants and children but could not protect seropositive children (19). To date, some clinical trials (20, 21) have been carried out on attenuated virus vaccines; there were some disadvantages such as the possibility of reversion of vaccine strain virus to the wild type (22) or appearance of symptoms (21). For example, in an investigation conducted by Karron on the invention of a live-attenuated vaccine for adults and human respirovirus-1-seropositive and seronegative children, despite the very limited replication of the virus, the symptoms of fever and sore throats occurred in two children and adults respectively (21). In addition, these vaccines should not be administered to pregnant women, immuno-compromised people and elderly individuals (23).

Some efforts were also made to develop protein subunit vaccines. On the one hand, only produced proteins could not induce a proper immune response and had to accompany by adjuvants. On the other hand, these vaccines were not cost-effective due to the necessity of using a large amount of antigens (24). Due to these problems, we concentrated on the progress of a DNA vaccine. Along with this, in the first step, pcDNA3 plasmids were used as the backbone to insert the synthetic HN gene downstream of the cytomegalovirus (CMV) promoter. The vector has CpG motifs that increase vaccine efficacy to trigger the immune responses. Western Blotting evaluated the COS-7 cell lines that transfected by Polyethyleneimine (PEI) and HN protein. Compared to other transfer approaches like cationic lipids (lipofection) and polymers, PEI has low toxicity. It works in the presence or absence of serum, does not elicit the immune response, and efficiently transfers the DNA (25, 26).

Liang and his colleagues reveal that the insertion of a partially modified F gene of the human respiratory syncytial virus into the rB/HPIV3 vectors, although tolerated, elicited sufficient neutralizing antibodies against RSV in animal models (27). In another study, HN and/or F gene of human respirovirus 3 were introduced into modified vaccinia virus Ankara.

On manipulating the responsible promoter of the expression of the HN and/or F gene, the levels of these proteins were not found to be toxic. When inoculated in cotton rats, they produced high levels of antibody (28). In the next step, we would like to examine this pcDNA3 plasmid in an animal model in order to evaluate the neutralizing antibodies and immunogenicity of the HN protein.

Conclusion

We prepared a DNA plasmid containing HN gene of human respirovirus 3 to be used for the further future studies to provide a vaccine.

Conflict of interest

None

Acknowledgment

This study was financially supported by Islamic Azad University-Rasht Branch, and the authors are grateful for their financial assistance.

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